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ALANINE UPTAKE BY ISOLATED ZOOXANTHELLAE OF THE MANGROVE JELLYFISH, *CASSIOPEA XAMACHANA*. I. TRANSPORT MECHANISMS AND UTILIZATION

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ABSTRACT

Freshly isolated zooxanthellae of the mangrove jellyfish *Cassiopea xamachana* were found to take up exogenous ^{14}C-alanine and incorporate the radiolabel into various cellular components. Transport was highly Na^+-dependent and lacked stereospecificity. Competition experiments with several L-amino acids and alanine analogs exhibited varying degrees of competitive inhibition, and these results are discussed in relation to the well described neutral amino acid transport systems known for many eukaryotic cells. Inhibition of uptake by KCN and N-ethylmaleimide indicated an active process and a significant electrogenic component to the energetics of uptake. Uptake of alanine was not affected by the inhibition of photosynthesis.

INTRODUCTION

Unicellular algae, termed zooxanthellae, occur in symbiotic relationships with a wide variety of marine invertebrates. Most of these associations occur in members of the phylum Cnidaria (Coelenterata), especially in hermatypic corals and sea anemones. Though the association is not an obligate one, the widespread occurrence and stability of such symbioses suggest that the metabolic relationships which exist between symbionts are well regulated and of mutual physiological advantage.

It is now well documented that the photosynthate produced by zooxanthellae can be translocated to animal host cells (for reviews, see Muscatine, 1974, 1980). In coral, these products are primarily glycerol with smaller amounts of glucose and alanine (Muscatine and Cernichiari, 1969; Lewis and Smith, 1971; Trench, 1974). Other studies on cnidarians suggest that significant amounts of lipid may also be translocated (Patton *et al.*, 1977; Blanquet *et al.*, 1979).

The quantity of organic material supplied to the animal may be considerable. von Holt and von Holt (1968) reported that in *Zoanthus* and *Condylactis* as much as 70% of the algal photosynthate may be transported in three hours. Trench (1971) found that about half of the radiolabeled carbon fixed by zooxanthellae of the sea anemone *Anthopleura* was transferred. Muscatine *et al.* (1981) recently estimated that the zooxanthellae of *Fungia* and *Pocillopora* can supply 63–69% of the daily respiratory carbon demand in these corals.

The back-transfer and utilization of animal metabolites by zooxanthellae, on the other hand, are not as well studied. Although Muscatine (1978) examined the uptake and retention of dissolved, inorganic nitrogen, little is known about the exchange of small organic molecules. Cook (1971) demonstrated the incorporation of isotope in the zooxanthellae of the anemone *Aiptasia* which had been fed ^{35}S-labeled mouse

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liver and suggested that the algae could exchange amino acids with host amino acid pools. Trench (1979) reported that when organic-^{35}S was fed to *Anthopleura,* up to 40% of the label was transferred from the anemone to the zooxanthellae. Thorington and Margulis (1981) also suggested that in green hydra, zoochlorellae and host compete for the available pool of organic compounds obtained from host feeding. They found that, with time, levels of radiolabeled metabolites decreased in the host as label increased in the algae. When hydra were maintained in the dark, algal cells could survive for months in the absence of photosynthesis. Thus, the ability to utilize host metabolites may considerably enhance the potential for algal survival and assure the stability of the association.

No investigations, however, have focused on the specific mechanisms by which such back-transfer occurs for any type of organic compound. This study was thus designed to characterize some aspects of amino acid uptake by freshly isolated zooxanthellae from the mangrove jellyfish *Cassiopea xamachana* and to compare these to the amino acid uptake mechanisms of other cell types. Alanine was chosen as a probe, since it is a common constituent of the free amino acid pools of eukaryotic cells, and characteristics of its uptake have been well studied. In addition, alanine has been shown to be an end-product of anaerobic metabolism in cnidarians (Ellington, 1977, 1980, and 1982), and thus may be produced by *Cassiopea* under the hypoxic conditions of the marine mangrove swamps which it inhabits.

Materials and Methods

Cassiopea xamachana were obtained from Marine Specimens Unlimited (Summerland Key, Florida) and maintained in the laboratory in filtered sea water (FSW) made from INSTANT OCEAN sea salts (Aquarium Systems, Eastlake, Ohio). All media were adjusted to pH 8.3 and a salinity of 29 ± 1 ppt. Animals were used within ten days of arrival.

For studies on sodium-specificity of amino acid uptake, artificial sea water and sodium-free medium were prepared according to the Woods Hole Formula (Cavanaugh, 1964). For sodium-free medium, appropriate amounts of choline chloride and $KHCO_3$, were substituted to maintain the same molarity of NaCl and $NaHC0_3$, respectively.

Isolation of zooxanthellae

Cassiopea zooxanthellae were freshly isolated for each experiment according to the methods of Blanquet *et al.* (1979). Final cell suspensions were checked for purity by light microscopy and standardized for all experiments at $3 \cdot 10^6$ cells ml^{-1}. Cell counts were determined by a hemocytometer. Levels of total chlorophyll, (chl a and c_2), expressed in μg chl ml^{-1}, were determined by the methods of Jeffrey and Haxo (1968). All experiments were performed under standard conditions of light (45 ± 3 μEinstein m^{-2} s^{-1}) and temperature (23 ± 2°C).

Radioisotope uptake studies

Chemicals. Radiolabeled L-(1-^{14}C)alanine (56.2 mCi $mmole^{-1}$), D-(1-^{14}C)alanine (56.2 mCi $mmole^{-1}$), L-(U-^{14}C)alanine (144 mCi $mmole^{-1}$), and (1-^{14}C)α-aminoisobutyric acid (AIB) (56.3 mCi $mmole^{-1}$) were purchased from ICN Biochemicals, Irvine, California. (1-^{14}C)β-alanine (54.7 mCi $mmole^{-1}$) and L-(U-^{14}C)alanine (174 mCi $mmole^{-1}$) were obtained from New England Nuclear, Boston, Massachusetts.

Non-radiolabeled amino acids, analogs, ouabain, and N-ethylmaleimide (NEM) were obtained from the Sigma Chemical Company (St. Louis, Missouri), DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethyl urea, was obtained from Dupont de Nemours and Company (Wilmington, Delaware).

Protocol. Algal cell suspensions were allowed to incubate under standard experimental conditions for one hour, with gentle agitation on a Model 3520 Orbit Shaker (Lab-Line, Melrose Park, Illinois), prior to the addition of radiolabeled compounds.

In all assays, ^{14}C-compounds were added to cell suspensions to obtain a final concentration of 1 μCi ml^{-1}. Immediately after the addition of labeled compounds, aliquots (0.1 ml) were removed to determine the initial level of radioactivity. Triplicate samples (1.0 ml) were subsequently removed at designated time periods and the zooxanthellae collected on Whatman GF/C filter discs using an Amicon Filtration Manifold. Discs were thoroughly washed, allowed to air dry, and placed in scintillation vials to which 10 ml of Aquasol (New England Nuclear) were added. Uptake values were expressed as DPM $\cdot$ μg chl^{-1}ml^{-1} and rates of uptake (slope) as uptake per hour. Samples were counted in a Beckman, Model LS7500, programmable liquid scintillation counter.

In experiments designed to monitor the competition of unlabeled amino acids and analogs with the uptake of L-(U-^{14}C)alanine, unlabeled substrates were added in 5000-fold concentrations to that of alanine. Metabolic inhibitors were used at the following concentrations: KCN, 0.01 m*M;* NEM, 0.5 and 5.0 m*M;* ouabain, 0.1 m*M;* NaF, 1.0 m*M;* and DCMU, 0.001 and 0.01 m*M.*

Distribution of incorporated alanine

To investigate algal utilization of alanine, zooxanthellae were incubated over three hour time periods in L-(1-^{14}C)alanine, washed free of exogenous isotope, and sequentially extracted in 50% ethanol followed by chloroform:methanol (3:1). Triplicate samples (0.4 ml), taken to dryness, were counted immediately after uniform algal suspension in each extractant and the dried supernatants counted to insure the completeness of the operation. Cell densities were maintained at uniform levels by appropriate dilution with each extraction solvent. Radioactivity remaining in the final pellet was determined by difference.

Statistics

Three-hour comparisons were used in this study, since standard linear regression techniques showed that uptake rates for alanine were linear over the course of six hours. Student's *t*-tests and regression analyses were carried out according to Snedecor and Cochran (1971).

Results

Rate of alanine uptake by zooxanthellae

Alanine uptake rates (DPM $\cdot$ μg chl^{-1}ml^{-1}h^{-1}) were determined by measuring the slopes for seven different algal cell suspensions during six hour incubation periods in L-(U-^{14}C)alanine. Uptake measurements were made after the first thirty minutes and after each hour.

Slopes for these experiments were found to be highly linear as indicated by the correlation coefficients (mean $\pm$ S.D. = 0.9836 $\pm$ 0.0247). A high degree of variability among the rates was noted, however (mean $\pm$ S.D. = 1171 $\pm$ 914).

Specificity of alanine uptake

Isolated zooxanthellae did not appear to exhibit stereospecificity of alanine uptake (Fig. 1). Comparisons of uptake of D-(1-^{14}C)alanine to that of L-(1-^{14}C)alanine ranged from 101–109% (n = 3), and the 3-hour mean values were not statistically different ($P > 0.10$). These results differ from those reported for various animal cells or purified plasma membrane vesicles where varying, but significant, differences were noted with different amino acids (Sigrist-Nelson *et al.*, 1975; Sacktor, 1977; Im and Spector, 1980; Kilberg *et al.*, 1980, 1981; Handlogten *et al.*, 1981).

The uptake rate for L-(1-^{14}C)alanine compared to the (1-^{14}C)analogs β-alanine and α-aminoisobutyric acid (AIB) is presented for a typical experiment in Figure 2. Both analogs showed highly reduced 3-hour uptake values compared to controls, ranging from 24–31% (n = 2) for β-alanine and 4–24% (n = 4) for AIB.

Ion-dependency of alanine uptake

Uptake of exogenous ^{14}C-alanine was found to be highly dependent on the presence of sodium in the medium. Uptake values after 3-hour incubation periods in sodium-free medium were 3.48 ± 2.57% of controls (n = 4). Thus, approximately 97% of alanine uptake by isolated zooxanthellae was sodium-dependent, which is consistent

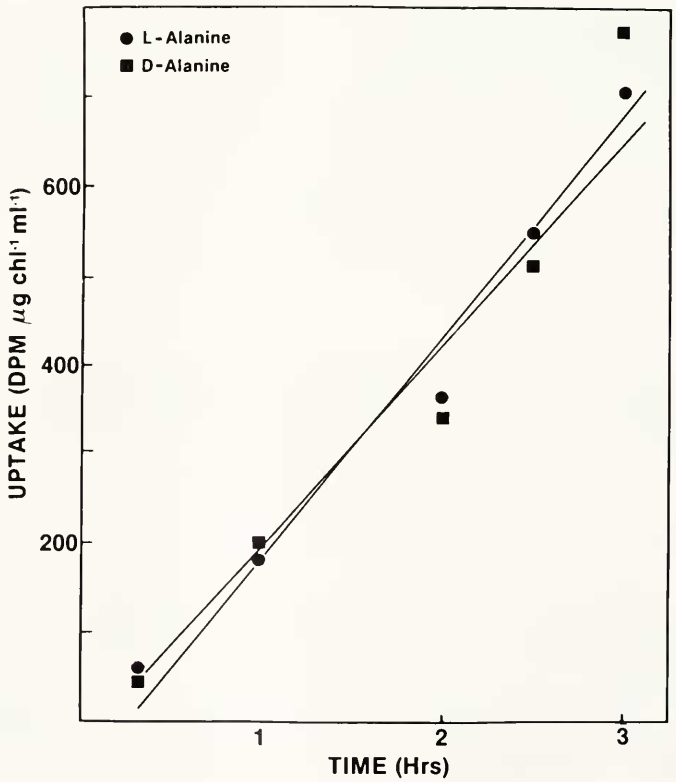

FIGURE 1. Uptake of L- and D-(1-^{14}C)alanine by isolated zooxanthellae.

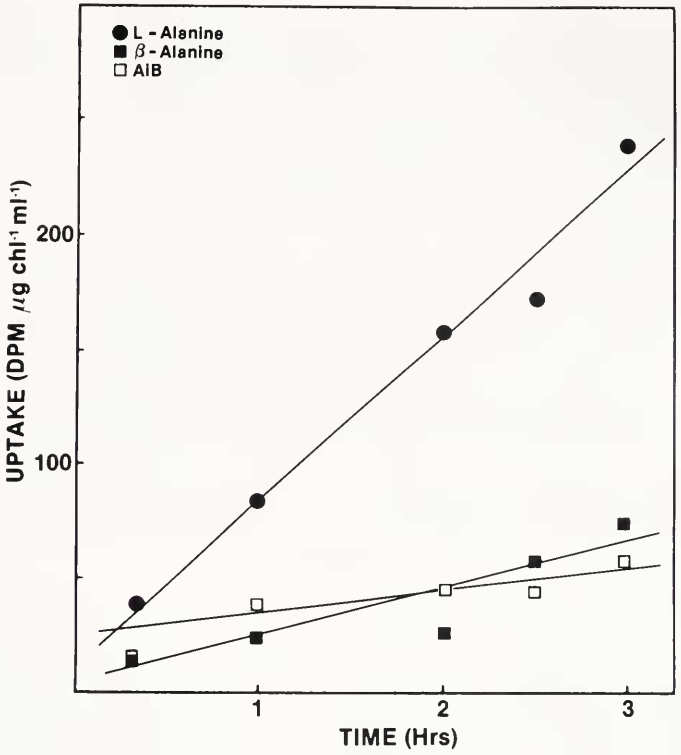

FIGURE 2. Uptake of L-(1-^{14}C)alanine, β-alanine, and α-aminoisobutyric acid (AIB) by isolated zooxanthellae.

with the results reported for this amino acid in a wide variety of cells (Guidotti *et al.*, 1978; Christensen, 1979, 1982).

Uptake systems for alanine in zooxanthellae

Sodium-dependent alanine uptake occurs in a variety of cell types via two systems designated A and ASC by Christensen (1979) and co-workers (Kilberg *et al.*, 1980, 1981; Handlogten *et al.*, 1981). System A functions primarily in the uptake of neutral amino acids with short, polar, or linear side-chains. A sub-set of System A, the ASC System, has the greatest affinity for three such amino acids: alanine, serine, and cysteine. Transport of an amino acid such as alanine by each of these systems can be distinguished by competition experiments using the methylated analog α-methylaminoisobutyric acid (MeAIB). This analog competes for uptake sites of System A but not of System ASC. In contrast to these systems, System L is sodium-independent and transports neutral aromatic amino acids or those that have branching side-chains.

Since these systems are characteristic of a variety of cell types, similar mechanisms in zooxanthellae may provide a basis for the interpretation of alanine uptake in the presence of the competing amino acids or analogs (Table I). The lack of a large reduction of alanine uptake in the presence of MeAIB (10% of control) would argue that the ASC System serves as the major uptake system for alanine in zooxanthellae. The predominance of the ASC System is also consistent with the reduction of alanine

TABLE I

Uptake of L-(U-^{14}C) alanine (174 mCi mmole^{-1}) by isolated zooxanthellae in the presence of 5000-fold molar excess of unlabeled amino acids and analogs

Compound	Mean percent ± S.D.
Control	100.0
L-alanine (4)	48.9 ± 4.2
MeAIB (3)	89.5 ± 24.7
L-serine (4)	65.5 ± 6.8
L-cysteine (3)	73.9 ± 15.3
β-alanine (3)	71.1 ± 1.7
L-valine (3)	88.0 ± 4.0
L-leucine (3)	92.2 ± 7.1

Values represent 3-hour uptake levels (DPM · μg chl^{-1} ml^{-1}), expressed as mean percentage ± S.D. of control levels. Number of experiments are given in parentheses. MeAIB = α-methylaminoisobutyric acid.

uptake in the presence of the amino acids which share this system, L-serine and L-cysteine. In contrast, amino acids transported by System L, such as L-valine and L-leucine, were not as effective as competitors.

Effects of inhibitors on alanine uptake

The effects of inhibitors on the uptake of alanine by isolated zooxanthellae is shown in Table II. Uptake of L(U-^{14}C)alanine was markedly depressed by KCN (0.01) and by NEM (0.5 and 5.0 m*M*), although a significant amount of alanine uptake still occurred in their presence. NEM has been used to inhibit ATPase activity in plants (Leonard, 1982), and ^{3}H-NEM has been used to analyze the role of sulfhydryl group integrity in the Na^+,K^+-ATPase of mammalian cells (Winslow, 1981). In contrast, the glycolytic inhibitor NaF had no apparent effect. The lack of reduction of alanine uptake in the presence of ouabain, a cardiac glycoside which is extremely effective in inhibiting animal ATPase enzymes, is noteworthy and may reflect differences between the ATPases of plants and animals. In this regard, ATPase insensitivity to ouabain has been reported in several algae and higher plants (Leonard and Hodges, 1973; Sullivan and Volcani, 1974; Hellebust, 1978; Leonard, 1982).

TABLE II

Effects of inhibitors on uptake of L-(U-^{14}C) alanine (144 mCi mmole^{-1}) by isolated zooxanthellae

Inhibitor	Inhibitor concentration (m*M*)	Percentage of control uptake (average)
None	—	100
KCN	0.01	45 (3)
N-ethylmaleimide	5.0	35 (2)
	0.5	27 (2)
NaF	1.0	95 (2)
Ouabain	0.1	110 (2)
DCMU	0.01	97 (4)
	0.001	111 (4)

3-hour uptake values = DPM μg chl^{-1} ml^{-1}. Number of experiments are given in parentheses. DCMU = 3-(3,4-dichlorophenyl)-1,1-dimethyl urea.

The effect of the photosynthetic inhibitor DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethyl urea, was tested to evaluate the role of photosynthetic production of amino acids on exogenous alanine uptake (Table II). *Cassiopea* zooxanthellae incubated in the presence of ^{14}C-bicarbonate and 0.01 m*M* DCMU for three hours demonstrated that less than 1% of the isotope was fixed. ^{14}C-alanine uptake, in the presence of this inhibitor, on the other hand, was not significantly reduced ($P > 0.10$), despite the almost complete shutdown of photosynthesis.

Distribution of incorporated alanine

The distribution of alanine in zooxanthellae exposed to L-(1-^{14}C) alanine for three hours shows that the algae are able to metabolize this amino acid into several types of compounds (Table III). Over half the radiolabeled material remained in the insoluble pellet after sequential extraction of the algal cells with ethanol and chloroform:methanol, indicating incorporation into high molecular weight compounds and structural components. Soluble compounds differentially extracted by ethanol and chloroform:methanol would be expected to contain low molecular weight organic and lipid components, respectively.

Discussion

While the translocation of organic nutrients from endozoic algae to their animal hosts is well documented, less attention has been paid to the extent of back-transfer of such molecules. Several studies (Cook, 1971; Thorington and Margulis, 1981) have provided evidence that endozoic algae may compete for the pool of soluble organic nutrients available from host metabolism, especially during periods of photosynthetic inactivity. Previous studies have not characterized the mechanisms involved in the transport of nutrients, such as sugars and amino acids, by zooxanthellae, so that they might be compared to similar systems in non-symbiotic unicellular algae or in animal cells. Studies such as these are essential to our understanding of the maintenance and regulation of these symbiotic associations.

The results of this investigation support the view that isolated zooxanthellae take up alanine by mechanisms similar to those of animal cells, and no systems unique to the symbiotic state appear to be operative. Incorporation studies (Table III) suggest that algal endosymbionts can utilize alanine, and possibly other nutrients present in animal cells pools, for growth and maintenance.

As demonstrated for a wide variety of cell types, uptake by zooxanthellae is highly

Table III

Distribution of ^{14}C in isolated zooxanthellae following 3-hour incubation periods in L-(1-^{14}C) alanine

Experiment	Solvent		
	Ethanol	Chloroform:methanol	Algal pellet
1	25.7	26.6	47.4
2	34.8	22.1	43.1
3	20.5	14.1	65.4
4	20.4	16.3	59.7
Mean ± S.D.	25.4 ± 6.7	19.8 ± 5.7	54.0 ± 10.4

Values represent percentage of total radioactivity extracted by the solvent or remaining in the final wash pellet, for 4 separate experiments. Totals are mean percent ± S.D.

Na^+-dependent. Although stereospecificity appears to be lacking, reduced uptake rates of alanine analogs demonstrate that there is transport specificity. This is further supported by competition experiments using several natural amino acids and analogs (Table II) which exhibited varying degrees of competitive inhibition. Such experiments may also be interpreted in terms of the systems of amino acid transport described in studies by numerous investigators on a variety of cells (Christensen, 1979; Kilberg *et al.*, 1980, 1981; Handlogten *et al.*, 1981; Shotwell *et al.*, 1981; Christensen, 1982). System ASC appears to be the primary transport mechanism for alanine since MeAIB, an inhibitor of System A but not ASC, was ineffective in suppressing uptake. This conclusion is also supported by the observations that serine and cysteine, which, along with alanine, are most effectively transported by System ASC, were the most effective competitors. Amino acids which are transported by the Na^+-independent System L, such as valine and leucine, did not significantly reduce uptake.

It should be noted that the ratio of these uptake systems varies widely among the many types of cells studied and may not remain constant within a given cell type. Adaptive changes in the activity of System A in animal cells have been demonstrated with respect to varying exogenous levels of relevant amino acids (Gazzola *et al.*, 1981; Shotwell *et al.*, 1981). Cells incubated under conditions of amino acid deprivation demonstrated increased System A activity, while those exposed to high levels of exogenous amino acids showed depressed uptake. These responses were not found for System ASC. Such adaptive responses, if present in zooxanthellae, could account for the variability of uptake rates seen in different algal cell suspensions. Variability may also be due to differences in *in situ* preconditioning by animal host factors as reported in the following paper (Carroll and Blanquet, 1984).

The question of whether uptake of exogenous alanine may be affected by the endogenous production of amino acids through photosynthesis was addressed in experiments utilizing the photosynthetic inhibitor DCMU. The results of these assays indicated that uptake of ^{14}C-alanine in the presence of DCMU was not significantly different from controls. In analogous investigations using animal cells, the uptake of amino acids was unaffected after preloading cells with these compounds in order to elevate artificially internal levels (Guidotti *et al.*, 1978).

Some features of alanine uptake by zooxanthellae, exemplified by the effects of inhibitors, were found to differ from animal cells. In their review, Kimmich and Carter-Su (1978) proposed a model in which differences in both the ionic and electrical potentials across plasma membranes play important roles in energizing the concentrative uptake of sugars and amino acids. While electrogenic potentials are generated by membrane ATPase activity, it should be noted that the properties of this enzyme differ in plants and animals. In general, higher plants and algae possess a K^+-ATPase, where the K^+ influx does not appear to be coupled to Na^+ efflux, as occurs in animal cells (Leonard, 1982). Other studies suggest that electrogenic extrusion of H^+ is a prominent feature of plant cells (Poole, 1978; Smith and Raven, 1979), and it is possible that the efflux of H^+, instead of Na^+, is coupled to K^+ transport. If the ATPase is purely an electrogenic proton pump, an antiport mechanism could facilitate an uphill Na^+ efflux. Such a mechanism has been identified in *Halobacterium halobium*, where a symport for Na^+-alanine uptake is coupled to an antiport for H^+ and Na^+ (Lanyi and MacDonald, 1976). Such a coupled mechanism could account for the ouabain-resistant, sodium-dependent uptake of alanine observed in zooxanthellae.

The effect on alanine uptake in zooxanthellae by exogenous KCN is also noteworthy (Table II). While KCN markedly depressed alanine uptake, a significant uptake occurred in its presence. A possible explanation is the continued production of ATP via a cyanide-resistant respiration pathway, which has been reported in some algae,

higher plants and animals (Lloyd, 1974; Henry and Nyns, 1975; Zaba, 1983). Such respiration occurs by an alternative electron transport route, which arises after the first oxidative phosphorylation site, and allows the transport of electrons to oxygen without further ATP production. The existence of such a pathway, however, has recently been questioned (Kelly, 1982).

There is increasing evidence suggesting that endozoic algae compete for and utilize nutrients available in animal cell pools. However, the extent to which the stability of symbiotic associations depends upon the regulation of such reciprocal transfers is not known. Investigations focusing on such questions are necessary for a fuller understanding of such symbioses.

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